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Assessment of genetic diversity of mints Iranian wild "*Mentha aquatic"* populations using RAPD marker

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Mentha aquatica is one of the most important medicinal plants. The first step for breeding purposes is to determine the genetic variation. There exists various accessions of this plant in Iran; however, no comprehensive study to fully understand it has ever been carried out. In this study, the genetic diversity of 51 Iranian wild populations of *Mentha aquatica* has been evaluated with random amplified polymorphic DNA (RAPD) markers. Total of 120 primers were screened from which 20 primers were selected for RAPD analysis. 20 primers were used that produced 240 bands. Among them 228 (93.94%) bands were polymorphic and were 12(6.06%) bands monomorphic. Cluster analysis of the genotypes was performed using Jaccard's similarity coefficient and UPGMA method and as a result 51 samples of Iranian *Mentha aquatica* were divided into 13 groups. The least and highest similarity coefficient were 0.21 and 0.79 respectively. The obtained dendrograms and groups showed that the applied markers in the research could distinguish the *Mentha aquatica* sample properly. Finally, investigation of genetic variation on this species indicated that RAPD marker is suitable approach to determine the polymorphic loci and to estimate the genetic distance between the populations of the species.

Key words: *Mentha aquatica,* RAPD, Genetic diversity, wild accessions, Iran

Introduction

Mints are herbaceous plants and perennial aromatic herbs that are cultivated for their essential oils used both for medicinal and culinary purposes (Gobert *et al.,* 2002). *Mentha aquatica* belongs to the genus *Mentha* L. (*Lamiaceae*), which is a native from north temperate regions and occur in all five continents, with a wide geographical distribution in Iran. *Mentha aquatica* is one of the most important medicinal plants. The leaves are anodyne, antiseptic, antispasmodic, astringent,carminative, cholagogue, diaphoretic,

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emetic, refrigerant, stimulant, stomachic, tonic and vasodilator (Launert 1981, Lust 1983, Grieve, 1984 ; Kizil *et al.,* 2005; Hemphill *et al.,* 2006). A tea made from the leaves has traditionally been used in the treatment of fevers, headaches, digestive disorders and various minor ailments (Foster and Duke 1990; Olotuah, 2006). It is also used as a mouth-wash and a gargle for treating sore throats, ulcers, bad breath and etc (Launert, 1981). The leaves are harvested as the plant comes into flower and can be dried for later use. The essential oil in the leaves is antiseptic, though it is toxic in large doses (Foster and Duke, 1990). Although, some attempts have been started in recent years for domestication of this plant, no effort has been made to analyze variability among different populations of this plant. Information on genetic variation of the available germplasm is fundamental to its domestication, improvement and management. This could also provide information on the evolving process and distribution of the germplasm in different isolated regions (Heywood, 2002). An understanding of the extent and organization of genetic diversity among these species could be useful for both its genetic improvement and conservation. It is difficult to distinguish phenotypically similar cultivars using morphological and physiological methods or isozyme analyses (Reynders and Bollereau, 1994).

The limitation of these analyses is that they are phenotypic based. Recently the development of polymerase chain reaction (PCR) technique has revolutionized the field of molecular biology (Waugh and Powell1992, Deshmukh *et al.,* 2009). An understanding of genetic diversity is essential to properly maintain and exploit germplasm resources and to develop a global strategy for better management and more effective use of variation in collected germplsam (Brown, 1989). The DNA fingerprinting technique of Randomly Amplified Polymorphic DNA (RAPD) provides an unlimited number of markers which can be used for various purposes (Williams *et al.,* 1990). RAPD markers can be generated using short arbitrary primers to amplify genomic DNA, giving a genotype-specific pattern of bands. RAPD analysis should lead to the saturation of the genome without the requirement of previous genetic information (Williams *et al.,* 1990). RAPD is the most widely used molecular marker for analysis of DNA fingerprinting. The RAPD technique has become an increasingly popular tool in genetic studies (Emadpour, 2009). Two attempts to assess genetic relationships Khanuja *et al.* (2000) and cultivar identity (Fenwick and Ward, 2001) based on RAPD markers have been undertaken in Mentha species. In this study, we used RAPD technique to distinguish DNA polymorphism and genetic diversity various accessions of this plant in Iran.

Materials and methods

Plant material

During September to October of 2008-2009, (or Sept. 2008 - Oct. 2009) 51 Wild populations of *Mentha aquatica* were collected from different regions in Iran. Seed all these accessions were grown in the same conditions in a greenhouse. Collection sites, population's codes and their major ecogeographical parameters are presented in Table 1.

Table 1. *Mentha aquatica* populations, their collection sites, codes, elevation, latitude, longitude and collection dates during the year 2008-2009

Number	Population Name/Collection Site	Population Code	Elevation (m)	Latitude East	Longitude North
1	Ilam A,B,C	1-3	1393	33.638	46.431
2	Khuzestan A,B,C	4-6	39	31.314	48.68
3	Kermanshah A,B,C	7-9	1389	34.314	47.065
4	Kurdistan A,B,C	10-12	1464	35.31	46.999
5	Bushehr A,B,C	13-15	9	28.974	50.834
6	Hamedan A,B,C	16-18	1824	34.795	48.514
7	Azarbaijan West A,B,C	19-21	1267	35.58	44.03
8	Golestan A,B,C	22-24	183	36.828	54.439
9	Gilan A,B,C	25-27	1	37.278	49.595
10	Mazandaran A,B,C	28-30	52	36.568	53.059
11	Azarbaijan East A,B,C	31-33	1395	38.08	46.292
12	Qazvin A,B,C	34-36	1290	36.262	50.017
13	Lorestan A,B,C	37-39	1147.8	33.26	45.17
14	Semnan A,B,C	40-42	1137	35.575	53.406
15	Tehran A,B,C	43-45	1149	35.672	51.424
16	Karaj A,B,C	46-48	1430	35.829	51.006
17	Chahar Mahal and Bakhtiari A,B,C	49-51	1800	31.09	49.28

DNA extraction and rapd amplifications

Seed accessions in a greenhouse were germinated and total genomic DNA was isolated from the very young of plants according to the protocol of Doyle and Doyle (1997). Genomic DNA was precipitated by isopropanol cold and finally washed with 76% ethanol and dissolved in 200 TE buffer. RNAase 3.5 (solution (10 mg/ml) treatment was given to remove RNA from the samples. Concentration and quality of each sample of DNA calculated from the Optical Density (OD) values at 260 and 280 nm wavelength and DNA was regarded as being of good quality when, the OD_{260}/OD_{280} ratio was near 1.9 and working

solutions of genomic DNA (25 ng µl $^{-1}$) were prepared in sterile water. For PCR analysis, twenty 10-mer primers were used (Table 2). PCR was performed in a thermal cycler (Bio-Rad model i-Cycler) in a total volume of 25 µl containing 25 ng of genomic DNA template, 1.25 U Taq polymerase, 1.25 mM of each dNTP , 10 pmol random 10-mer primer, 1.5 Mm MgCl2, 1X PCR buffer (10 mM Tris-HCl, pH 8.0, 50 mM KCl, 1.5 mM MgCl2). Amplifications were performed in a DNA Thermo-cycler programmed as follows: an initial denaturizing at 94°C for 3 min followed by 35 cycles at 94°C for 1 min, annealing at 37 °C for 1 min and extension at 72 °C for 2 min, followed by one final extension at 72°C for 4 min. Finally, the samples were either held at 4°C for direct use or stored at -20°C until needed. After amplification PCR product was resolved by electrophoresis in 1/2% agarose gel for 2.16 h at 70 V with IX TBE buffer. Bands were visualized by staining with ethidium bromide (0.5 µg mL^{1}) under UV light (Figure 1). The Band size was determined with the DNA size marker (SM1553 Fermentas)

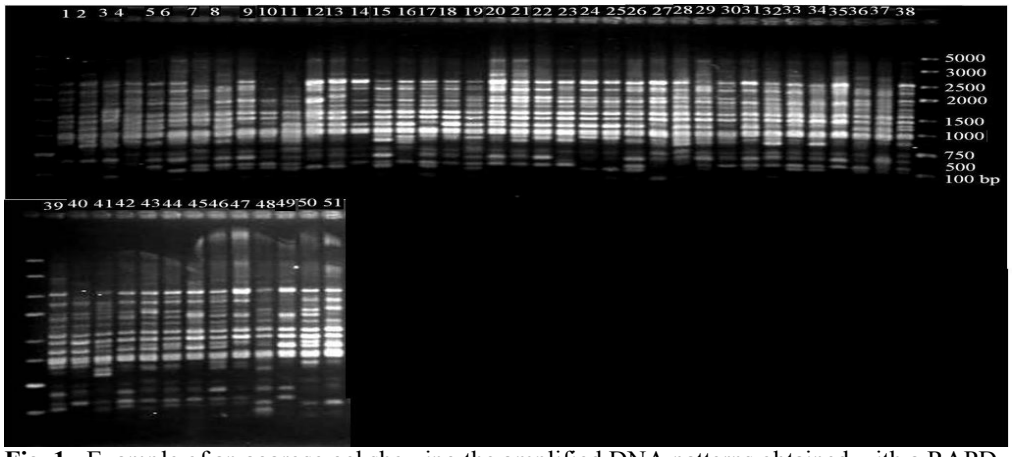

Fig. 1. Example of an agarose gel showing the amplified DNA patterns obtained with a RAPD-PCR reaction with primer R7

Data scoring and analysis

For each genotype, the presence and absence of fragments were scored as 1 or 0, respectively. Similarity index was estimated using the jaccards coefficient. Dendrograms were drawn using NTSyS-pc Version 2.02 softwares (Rohlf 1977; Jaccard, 1908). Cluster analysis was performed using the unweighted pair-group method with an arithmetic mean (UPGMA).

Results

Total of 120 primers (40 primer of TibMolBiol and 80 primer of NAPSE set) were screened from which 20 primers (10 primer of TibMolBiol and 10 primer of NAPSE set) were selected for RAPD analysis (Table2). 20 primers were used that produced 240 bands. Pic=1- X (Pi/Pn)^2

Pi: Number of alleles per row
Pn: Total number of alleles for each primer.

Table 2. Sequences of 20 primers and Polymorph bands and Polymorph rate of *Mentha aquatic*

Primer code	Nuclcotidc sequence	Total	pic	Poly bands	Poly rate
B1	TCACGCAGTT	17	0.65	17	100
B2	GGAAGGCTGT	10	0.94	8	80
R6	CATGTGCTTG	8	0.9	7	87.5
J7	ACATTGGGCG	14	0.96	12	85.71
J3	TGCTAGCCTC	12	0.82	12	100
R7	TCGGGATATG	15	0.71	12	80
R8	CACGGCGAGT	14	0.91	14	100
J12	CATGTGCTTG	11	0.72	11	100
J15	ATG ACGTTGA	10	0.84	9	90
JI6	ACATTGGGCG	13	0.77	13	100
BD13	CCTGGAACGG	14	0.32	14	100
BB08	TCGTCGAAGG TCGTCGAAGG	12	0.67	12	100
BD05	GTGCGGAGAG	7	0.74	6	85
BD15	TGTCGTGGTC	9	0.87	8	88.88
BA16	CCACGCATCA	15	0.9	15	100
BB11	TGCGGGTTCC	10	0.69	10	100
BE06	AAGCGGCCCT	13	0.78	13	100
BD17	GTTCGCTCCC	16	0.72	17	100
BB09	AGGCCGGTCA	9	0.89	9	100
BD04	TCGGGTGTTG	11	0.88	9	81.81
Total		240 bands	15.68	228 bands	
Mean		12	0.78	11.14	93.94

This value according to formula above for each of bands can be produced between one and zero is variable. Total resolving power (Pic) was 15.78 and J07 primer showed most resolving power equal to 0.96. The lowest similarity

(0.21) between khuzestan A; Ilam B; Mazandaran A and Golestan B genotypes and highest (0.79) detected between Khuzestan A; Kurdistan A; Hamedan A , Kohgiluyeh and Boyer Ahmad A . Cluster analysis based on jaccards similarity coefficients and UPGMA method and at similarity level of 0.54, were divided the genotypes into 13 sub-clusters which Tehran B; Golestan A, B, C; Gilan A; Mazandaran A, B, C genotype was separated individually from others at distance of 0.37. Cluster analysis resulted in grouping of the 51 *Mentha aquatica* accessions into nine main groups in 0.50 distance unit (Fig. 2). Eight genotype of the 51 accessions were included in first cluster; three accessions were in second cluster ; Fifth accessions in third cluster; fifteenth accessions in Fourth cluster ; Two accessions in Fifth cluster ;three accessions in Sixth cluster; Twelve accessions in Seventh cluster ;one accessions in eighth cluster and Two accessions in ninth cluster. Our result showed that There was little relationship between genetic divergence and geographical origins, so that the populations from similar geographical places (such as Ilam A and Ilam B) belonged to separate clusters. Conversely, populations from different geographical conditions (such as Ilam A and Bushehr C) relatively tended to be clustered in one subgroup of the dendrogram. These results show that there is high genetic diversity in Mentha aquatica .this high genetic diversity is good to the breeding to select excellent cultivars from the wild populations and can select new ones with high value and strong adaptation by crossing with different advantages and different habitation.

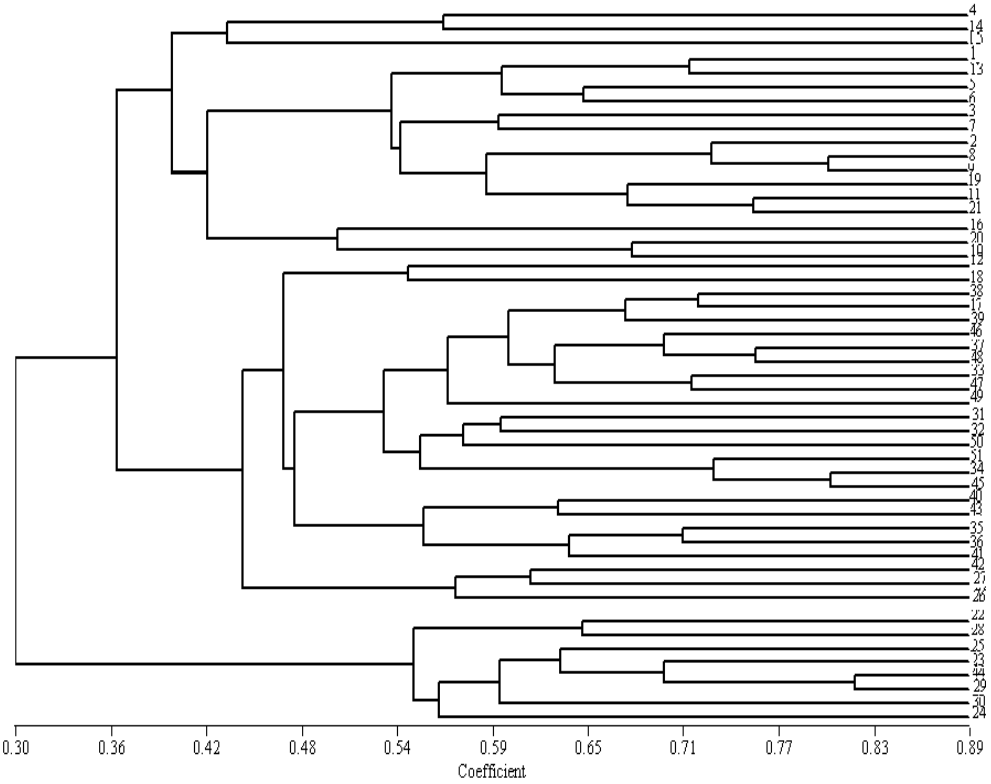

Fig.2. Dendrogram obtained from RAPD data of 51 accessions of *Mentha aquatica* by UPGMA

Discussion

The High diversity of *Mentha aquatica* is the reflection of adaptation to environment, which is beneficial to its propagation. *Mentha*, a taxonomically complex section, was recently studied by Khanuja *et al.* (2000) by RAPD fingerprinting. Genetic diversity and genetic relationships were assessed among six *Mentha* taxa (*M. arvensis*, *M. spicata*, *M. spicata* cv. *viridis*, *M.* x *piperita*, *M.* x *piperita* cv. *citrata*, *M.* x *gracilis* Sole cv. *cardiaca*). Effectively, new cultivars are selected as either spontaneous or induced variants or "sports" of existing cultivars and then clonally propagated (Gobert *et al.,* 2002). The lack of genetic diversity could result, for example, in a poor disease resistance as demonstrated by the widespread occurrence of verticillium wilt (caused by *Verticillium dahliae* Kleb.) (Fenwick and Ward, 2001; Akcin and Ozbucak, 2006). wild species represent the natural gene pool available for the genetic improvement of the cultivated *Mentha aquatic* It will be worth to investigate specific traits in the wild species and they may be introgressed by sexual crossing or somatic hybridization into commercial varieties (Singh *et al.,* 2006).

Generally, select parents with a higher general combining ability and long genetic distance can produce a hybrid with better yield performance (Shamsuddin, 1985). But the identification of combining ability based on morphological characters is costly and time-consuming and may be influenced by environment factors. In contrast, molecular markers are not directly influenced by environmental effects or epistatic interactions and can provide large numbers of loci.These results show that RAPD is suitable for genetic diversity assessment in *Mentha aquatica* .Classification of diversity in germplasm collections is important for both plant breeding and germplasm collection (Hadian *et al.,* 2007). Genetic similarity values are always difficult to compare with other studies because genetic variability depends heavily on factors including the history of the species, the reproductive system and ecology (Hamrick, 1989; Odukoya *et al.,* 2007).The RAPD analysis has been found to be a valuable DNA marker system to evaluate genetic diversity. The information about genetic similarity will be helpful to avoid any possibility of elite germplasm becoming genetically uniform. The information gathered here would be helpful in genomic mapping studies and for the development of *Mentha aquatica* accessions with wider and diverse genetic background to obtain improved plant productivity. These findings also proved that Iran is important center of *Mentha aquatica* diversity and *Mentha aquatica* germplasm of the Iran is very diverse. Detection of genetic differences and discrimination of genetic relationship between *Mentha aquatica* species are for utilization of plant genetic resources. Our study clearly indicated that RAPD markers could be effectively used for genetic diversity studies among accessions of *Mentha aquatica* native to Iran. Species-specific markers can be identified that would be useful for introgression studies where plant breeders want to transfer some desirable traits from one species into another. Localization of these markers on the chromosomes would be useful for keeping track of important traits that need to be transferred(Umamaheswari and Govindan, 2007). Genetically distinct cultivars were identified that could be potentially important sources of germplasm for *Mentha aquatica* improvement. The results obtained suggested that by using RAPD molecular markers the newly evolved *Mentha aquatica* can be easily differentiated from their parents. This would be a useful tool in identifying and protecting them from possible infringements in future. This assessment is fundamental because genetic diversity could be in future exploited through molecular approaches or plant breeding techniques to improve mint cultivars for disease resistance or to increase essential oil yield, for example (Gobert *et al.,* 2002).

Conclusion

Species-specific markers can be identified that would be useful for introgression studies where plant breeders want to transfer some desirable traits from one species into another. Localization of these markers on the chromosomes would be useful for keeping track of important traits that need to be transferred. Genetically distinct cultivars were identified that could be potentially important sources of germplasm for *Mentha aquatica* improvement. The results obtained suggested that by using RAPD molecular markers the newly evolved *Mentha aquatica* can be easily differentiated from their parents. This would be a useful tool in identifying and protecting them from possible infringements in future.

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